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Differential Regulation of Human Monocyte-Derived TNFα and IL-1β by Type IV cAMP-Phosphodiesterase (cAMP-PDE) Inhibitors

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ABSTRACT

Elevation of cAMP downregulates certain functions of inflammatory cells, including the release of TNFα and IL-1β by macrophages. Intracellular cAMP levels can be modulated pharmacologically by adding cell-permeable cAMP analogs, by stimulating adenylate cyclase or by inhibiting degradation of cAMP by cAMP-phosphodiesterases (cAMP-PDE). Multiple forms of cAMP-PDEs have been identified in various tissues and cells using both biochemical characterization and selective inhibitors. Therefore, we wanted to determine which of these different PDE isoforms was present in human monocytes and whether this isoform could regulate cytokine release from human monocytes by a mechanism similar to that seen with dbcAMP or PGE_1. Our results demonstrate that selective inhibitors of type IV cAMP-PDE, such as rolipram and Ro20–1724, are clearly the most effective compounds at enhancing cAMP levels and inhibiting the release of TNFα and IL-1β in these cells. The type III cAMP-PDE-selective inhibitors CI930 and cilostamide and the nonselective PDE inhibitors IBMX and pentoxifylline were significantly less potent. In agreement with these data, cAMP-PDE activity in cytosolic extracts from human monocytes was also much more sensitive to inhibition by rolipram than by cilostamide. Additionally, rolipram dramatically reduced TNFα mRNA accumulation, which supports previous findings that cAMP regulates TNFα at the transcriptional level. Surprisingly, rolipram, dbcAMP or PGE_1 increased IL-1β mRNA levels even though secretion of IL-1β was reduced, which indicates that cAMP can have both positive and negative effects on the regulation of IL-1β. Our results suggest that isotypes of type IV cAMP-PDE could provide a useful target for therapeutic intervention in inflammation and other diseases where TNFα and IL-1β are involved in their etiology.

Activated monocytes and macrophages produce and secrete IL-1β and TNFα in response to a variety of soluble and particulate stimuli (Sisson and Dinarello, 1989; Wong and Goeddel, 1989). These cytokines mediate chronic and acute inflammatory responses and are therefore important therapeutic targets in rheumatoid arthritis, septic shock and other inflammatory diseases (Hopkins, 1990). cAMP plays an important role in the regulation of these cytokines by modulating their release into inflamed tissues (Brandwein, 1986; Renz *et al.*, 1988).

Evidence that cAMP inhibits production of TNFα by LPS-stimulated murine peritoneal exudate macrophages or human monocytes and related cell lines has been obtained by treating these cells with dbcAMP or with prostaglandins to stimulate adenylate cyclase (Renz *et al.*, 1988; Scales *et al.*, 1989; Endres *et al.*, 1991). Either of these treatments reduces the amount of detectable mRNA for TNFα, which indicates that cAMP regulates this cytokine at the transcriptional level (Scales *et al.*, 1989). Similar results are reported when cAMP levels are elevated by exposing these cells to nonspecific inhibitors of cyclic nucleotide PDEs such as theophylline, IBMX or pentoxifylline (Endres *et al.*, 1991). Studies with these PDE inhibitors, however, only reported changes in secreted TNFα without measuring mRNA levels. In addition, many of the effects seen after treatment with methylxanthine-based PDE inhibitors can be attributed to factors other than changes in cAMP levels (Krzanowski and Polson, 1988).

Conflicting reports have been published regarding the inhibition of IL-1 production by cAMP-regulating compounds. Some authors report that total IL-1 production is not inhibited in human mixed mononuclear cells or adherent monocytes after treatment with PGE_2 or nonspecific PDE inhibitors (Endres *et al.*, 1991; Bailly *et al.*, 1990). On the other hand, indomethacin is often added to prevent synthesis of

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ABBREVIATIONS: PDE, phosphodiesterase; LDH, lactic dehydrogenase, ELISA, Enzyme linked immunosorbent assay; TNFα, Tumor necrosis factorα; IL-1β, Interleukin-1β; IBMX, 3-methyl-1-isobutyl xanthine; RIA, Radio immuno assay; DMSO, dimethylsulfoxide.

prostaglandins to enhance production of IL-1 (Dinarello *et al.*, 1987). More recent studies report that dbcAMP and PGE_2 reduce the amount of secreted IL-1β from LPS-stimulated adherent monocytes without affecting steady-state mRNA levels or cell-associated IL-1β (Knudsen *et al.*, 1986; Viherluoto *et al.*, 1991). These conflicting observations may arise from the use of bioassays rather than immunologically based ELISA or RIA assays to quantitate IL-1β or from differences in cell preparations and culture conditions. Nevertheless, the undisputed negative effects of cAMP-elevating agents on TNFα production and the possible downregulation of IL-1β secretion suggest that enzymes regulating intracellular cAMP levels provide good targets for modulating monocyte activation.

At least five different families of PDE enzymes, each with multiple members, have been identified (Beavo and Reifsnyder, 1990; Nicholson *et al.*, 1991; Silver, 1989). These enzymes differ in 1) substrate selectivity for cAMP *vs.* cGMP, 2) regulation by Ca^{2+}/calmodulin, cGMP or transducin, 3) tissue and subcellular distribution, 4) physical characteristics and 5) sensitivity to selective PDE inhibitors. The type I enzymes are regulated by calmodulin and do not discriminate well between cAMP and cGMP. On the other hand, type V PDEs are very selective for cGMP and are abundant in the retina and in platelets. PDEs with high affinity for cAMP are grouped as cGMP-inhibited type III or cGMP-resistant type IV PDEs on the basis of their sensitivity to cGMP. Type IV PDEs are very selective for cAMP, but type III PDEs degrade cAMP or cGMP with similar kinetics.

Although most tissues and cells can express multiple forms of PDEs, recent studies have demonstrated that human neutrophils and basophils contain predominantly type IV cAMP-PDE. Their activation is severely dampened by selective inhibitors of type IV (rolipram, Ro20–1724) but not by inhibitors of type III cAMP-PDE (CI930, cilostamide, milrinone) or by inhibitors of type V cGMP-PDE (zaprinast) (Verghese *et al.*, 1989; Nielson *et al.*, 1990; Peachell *et al.*, 1992). It has also been shown that type IV cAMP-PDE is present in monocytic cells (Thompson *et al.*, 1976; Torphy *et al.*, 1992) and that rolipram is also much more effective than CI930 in reducing TNFα secretion from human monocytes (Molnar-Kimber *et al.*, 1992; Semmler *et al.*, 1993). However, these reports did not address the mechanism by which rolipram affects monocyte activation. Such studies are important because rolipram and other PDE IV inhibitors exhibit significant discrepancies between their nanomolar affinity for binding and their micromolar IC_{50} values for inhibition of PDE IV activity (Lowe *et al.*, 1991; Torphy *et al.*, 1992). In the current study, we investigated whether inhibition of cytokine secretion by inhibition of PDE was mediated through increases in cAMP levels. Therefore, we compared the effects of a series of selective PDE inhibitors on cAMP levels, on cytokine production and on cytokine mRNA levels in intact monocytes and determined the pharmacological profile of monocyte cAMP-PDE activity. Our studies demonstrate that monocytes resemble other circulating inflammatory cells in that type IV cAMP-PDE appears to be the functionally predominant PDE to regulate cAMP degradation. Inhibitors of cAMP-PDE IV appear to regulate TNFα and IL-1β production by mechanisms similar to those utilized by dbcAMP and by PGE. This suggests that selective inhibitors of type IV cAMP-PDE could be potent pharmacological agents for the treatment of diseases in which the release of TNFα and IL-1β from human monocytes is thought to be important for their etiology.

Materials and Methods

Isolation and culture of human monocytes. Heparinized (10 U/ml) venous blood obtained from healthy volunteers was diluted ½ with pyrogen-free phosphate-buffered saline (Gibco, Grand Island, NY) containing 5 mM EDTA (PBS-E). Mononuclear cells were then separated by sedimentation over lymphocyte separating medium (Organon Technica, Durham, NC) and further purified by elutriation as described previously when large numbers of monocytes were needed for PDE assays (Verghese *et al.*, 1986). For cAMP and cytokine assays, monocytes were enriched by adhering cells for 30 min in 96-well flat-bottom plates (Falcon 3072) under normal tissue culture conditions in RPMI1640 supplemented with penicillin/streptomycin and 2.5% heat-inactivated pooled human AB serum. Nonadherent cells were washed off by vigorously rinsing each well two times with warm PBS. This procedure resulted in >80% peroxidase-positive cells. The wells were replenished with the same culture medium

Measurement of cAMP and cytokines. Adherent cells were exposed to test compounds or vehicle (DMSO) for 15 min before stimulation with 1 μg/ml of *E. coli* 0111:B4 LPS (Sigma L-2630). For cAMP measurements, plates were frozen in liquid nitrogen after a 15-min treatment with LPS to reflect initial levels of cAMP, which typically peak at this time (Serkkola *et al.*, 1992). We quantitated total cAMP levels (extra- and intracellular) with a scintillation proximity assay (Amersham, Arlington Heights, IL) after lysing the cells with 0.1% Triton X-100. All samples were done in triplicate and were repeated with at least three separate donors. The data were evaluated for statistical significance by analysis of variance (Snedecor, 1956). For cytokine assays, LPS stimulation was continued for 18 hr, and then the plates were centrifuged and half of the supernatant was removed to measure secreted IL-1β, TNFα or IL-8. Where indicated, cell-associated IL-1β was assayed after disrupting cells with three freeze-thaw cycles (Dinarello *et al.*, 1987). Cell damage was assessed by measuring release of LDH in the fresh supernatants with a Sigma kit (St. Louis, MO). IL-1β was measured by a Cistron ELISA kit (Pine Brook, NJ), and TNFα and IL-8 were measured by RandD Systems ELISA kits (Minneapolis, MN). The limits of detection for the IL-1β, TNFα and IL-8 kits were 20 pg/ml, 10 pg/ml and 5 pg/ml, respectively.

Northern blot analysis of mRNA from peripheral blood monocytes. Poly A+ RNA was isolated from 2-hr-treated and from untreated monocytes using the MicroFasttrack kit (Invitrogen Inc., San Diego, CA) according to the manufacturer's instructions. Approximately 1 μg of Poly A+ RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel and blotted to a nitrocellulose filter in 10× SSC (1.5 M NaCl, 0.15 M Na_3citrate, pH 7.0). The filter was baked at 80°C for 2 hr, prehybridized in 50% formamide, 4× SSC, 50 mM $NaPO_4$, 0.5% SDS, 1× Denhardt's reagent (Chirgwin *et al.*, 1979; Sambrook *et al.*, 1989) overnight at 37°C and hybridized overnight at 37°C to a specific oligonucleotide probe for TNFα (5' CGC CTC CTC GGC CAG CTC CAC GTC CCG GAT CAT GCT TTC AGT GCT CAT 3') or IL-1β (5' GCC ACT GTA ATA ACG CAT CAT TTC ACT GGC GAG CTC AGG TAC TTC TGC CAT 3') labeled with ^{32}P-dATP using terminal deoxynucleotidyl transferase (Gibco-BRL). The blots were washed twice with 0.1× SSC, 0.1% SDS at 55°C for 30 min and exposed to X-ray film. Radioactive bands were quantitated with a radioanalytical imager (AMBIS Co., San Diego, CA), and the resulting values were normalized to values obtained after hybridization to a ^{32}P-labeled, β-actin-specific oligonucleotide probe (5' CAT GCC GGA GCC GTT GTC GAC GAC GAG CGC GGC GAT ATC ATC ATC CAT 3'). The normalized values were plotted as a percent maximum *vs.* time.

Measurement of PDE activity. Elutriated monocytes (80%-90% peroxidase-positive) were washed and resuspended to 5 × 10^7

cells/ml in lysis buffer (20 mM Tris pH 7.3, 100 mM NaCl, 5 mM EGTA, 2 mM EDTA, 10 μg/ml each of leupeptin and aprotinin, 0.7 μg/ml pepstatin, 100 μg/ml soybean trypsin inhibitor, 100 μM "Pefabloc," 0.05% digitonin). Tubes were kept on ice for 5 min and then centrifuged for 5 min at 13000 × *g*. Supernatants were diluted before PDE activity was determined by a modification of the two-step reaction using 5' nucleotidase to convert 5' AMP to adenosine (Thompson *et al.*, 1976). Assays (50 μl final volume) contained 2 mM Tris pH 7.5, 1 mM MgCl$_2$, 100 μM EDTA, 330 μg/ml metal-free BSA, 10 μg/ml 5'-nucleotidase, 0.4 μCi ^{3}H cAMP (0.28 μM cAMP), 0.004 μCi ^{14}C adenosine, 10 μM of inhibitor or vehicle (DMSO) and 5 μl of diluted enzyme. Incubations were initiated by adding the radioisotopes and were terminated after 30 min at 30°C by transferring the samples to a Millipore 96-well filter plate containing 200 μl of a 50% slurry of QAE-Sephadex A-25 in 10 mM Hepes pH 7. After mixing for 5 min, the filtrate was collected and aliquots were mixed with scintillation cocktail and counted for dual label. The resulting values from an average of triplicate determinations were adjusted for differences in recovery and expressed as pmol cAMP/min/mg protein.

Materials. The following drugs and chemicals were kindly provided by or obtained from the sources indicated: PGE$_1$, dbcAMP, IBMX, 5'-nucleotidase and digitonin (Sigma Chemical Co., St. Louis, MO), 4-(2-aminoethyl) benzenesulfonyl fluoride ("Pefabloc") (Boehringer Mannheim, Indianapolis, IN), QAE-Sephadex (Pharmacia, Uppsala, Sweden), rolipram (Schering AG, Bloomfield, NJ), Ro20–1724 (BioMol Research Laboratories, Plymouth Meeting, PA), CI930 (Parke-Davis, Ann Arbor, MI), cilostamide and CP-77,059 were synthesized in house (Glaxo Medicinal Chemistry), tissue culture media (endotoxin-tested) and human serum (Gibco-BRL, Gaithersburg, MD) and radioactive reagents (New England Nuclear, Wilmington, DE).

Results

Effects of dbcAMP and of PGE$_1$ on release of TNFα, IL-1β and IL-8 from human monocytes. In order to study the effects of selective PDE inhibitors on the production of TNFα and IL-1β, we first needed to confirm whether increases in cAMP levels inhibited the release of these cytokines from adherent human monocytes cultured in the presence of pooled human AB serum (see "Materials and Methods"). Under these conditions, the addition of increasing concentrations of dbcAMP or PGE$_1$ progressively reduced the secretion of TNFα and IL-1β in response to 1 μg/ml of LPS (fig. 1). The corresponding increases in cAMP levels in response to PGE$_1$ ranged from 30% to 200%. The relative levels of cAMP after treatment with 0.01, 0.1 and 1 μM PGE$_1$ were 1.3 ± 0.1, 1.9 ± 0.6 ($P < .05$) and 3.0 ± 1.2 ($P < .05$), respectively, times the levels observed in untreated controls (202 ± 35 fmol/10^6 cells). Secretion of TNFα was completely inhibited by either dbcAMP or PGE$_1$, whereas IL-1β secretion appeared to be partially refractory and could never be reduced by more than 80%. Interestingly, secretion of IL-8 remained unaffected, which indicated that not all cytokine secretion was subject to modulation by cAMP and that inhibition was not due to a nonspecific toxic effect on the cells. Lack of toxicity was also confirmed by assaying LDH in the supernatants (data not shown).

Comparison of the effects of nonselective and selective PDE inhibitors on monocyte cAMP levels and on the secretion of TNFα and IL-1β by human monocytes. In order to determine which PDE inhibitors could regulate the release of cytokines from LPS-stimulated human monocytes, we compared the effects of nonselective PDE inhibitors, such as IBMX, theophylline and pentoxifylline to the effects of a series of inhibitors with selectivity toward type III or type IV cAMP-PDE. PDE inhibitors were tested at concentrations ranging from 10 nM to 100 μM. The resulting IC$_{50}$ values for inhibition of TNFα and IL-1β are shown in table 1 (columns 3 and 4, respectively), together with IC$_{50}$ values for cAMP-PDEs as reported in the literature (table 1, columns 1 and 2; see Beavo and Reifsnyder, 1990; Nicholson *et al.*, 1991; Silver, 1989; Lowe *et al.*, 1991).

Of the nonspecific PDE inhibitors, IBMX was more effective at inhibiting cytokine release than either theophylline or pentoxifylline. In general, these findings correlate with the reported rank order for inhibition of cAMP-PDE by these nonselective compounds. IBMX affected the secretion of TNFα more than the secretion of IL-1β (see table 1, parenthetical values for % inhibition at 100 μM). This result is consistent with the observed effects of dbcAMP and of PGE$_1$ on cytokine secretion (see fig. 1).

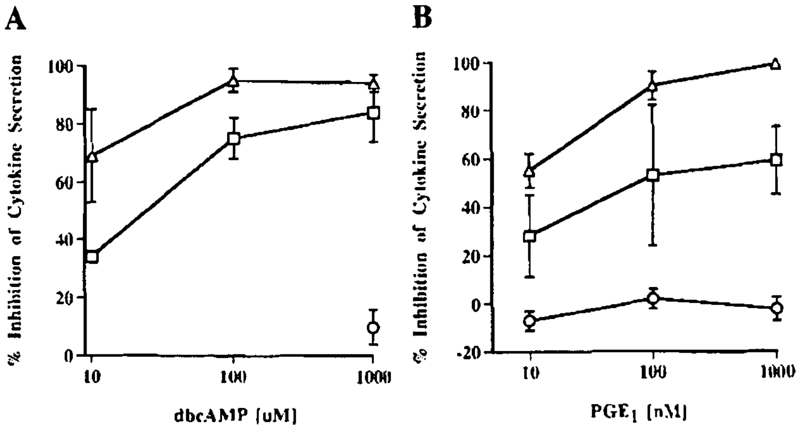

Fig. 1. Effect of dbcAMP and of PGE$_1$ on secretion of IL-1β, TNFα or IL-8 in LPS-stimulated human monocytes. Monocytes were isolated by adhesion in microtiter plates (200,000 peroxidase positive cells/well) and treated with dbcAMP (A) or PGE$_1$ (B) at the concentrations indicated before addition of 1 μg/ml of LPS. Supernatants were collected after 18 h, and levels of TNFα (△), IL-1β (□), or IL-8 (○) were determined by ELISA. Basal values from unstimulated control cells were at or below the detection level of the ELISA kits. LPS-stimulated values for 10^6 monocytes in the absence of other treatment were 2.9 ± 1.3 ng for TNFα, 6.7 ± 0.6 ng for IL-1β and 255 ± 83 ng for IL-8. Data presented are the mean % inhibition ± S.E.M. from three (IL-8) or five (IL-1β, TNFα) separate donors. Inhibition of TNFα was statistically significant ($P < .05$) at all concentrations of dbcAMP and of PGE$_1$. Inhibition of IL-1β was statistically significant ($P < .05$) at all concentrations of dbcAMP and at 1 μM PGE$_1$.

TABLE 1
Inhibition of TNFα or IL-1β by PDE inhibitors[a]

Inhibitor	IC_{50} in μM			
	Reported for Type III PDE Activity	Reported for Type IV PDE Activity	Measured for TNFα Secretion[b]	Measured for IL-1β Secretion[b]
Nonselective				
IBMX	2	15	15.9 (96%)	~100 (53%)
Theophylline	142	—	>100 (43%)	>100 (−13%)
Pentoxifylline	—	—	81.5 (71%)	>100 (7%)
Type III				
Cilostamide	0.03	40	100 (51%)	>100 (206%)
CI930	8	90	14.4 (74%)	>100 (20%)
Type IV				
CP-77,059	—	0.04	0.05 (98%)	0.2 (73%)
Rolipram	52	2.2	0.2 (97%)	4.5 (70%)
Ro20–1724	150	9	1.9 (96%)	4.5 (70%)

[a] Adherent monocytes were treated with PDE inhibitors (concentration range 0.01–100 μM) and TNFα and IL-1β was measured by ELISA (see "Materials and Methods"). LPS-stimulated TNFα and IL-1β levels were 2.1 ± 0.6 (n = 17) and 5.3 ± 0.4 (n = 17) ng per 10^6 cells, respectively. Columns 2 and 3 are representative IC_{50} values of enzyme inhibition for type III and type IV cAMP-PDE (Beavo and Reifsnyder, 1990; Nicholson *et al.*, 1991; Silver, 1989). IC_{50} values for inhibition of TNFα and of IL-1β are shown in columns 4 and 5, and maximum % inhibitions observed at 100 μM compound are shown in parentheses.

[b] Parenthetical values are % inhibition of cytokine secretion at 100 μM compound.

The two type III cAMP-PDE inhibitors tested, cilostamide and CI930, were ineffective at inhibiting TNFα and IL-1β secretion. Cilostamide inhibited the release of TNFα incompletely and actually enhanced the release of IL-1β (see table 1), even though it is one of the most potent inhibitors of type III cAMP-PDE. CI930 was more effective than cilostamide at inhibiting TNFα and IL-1β secretion in spite of its higher IC_{50} value for type III cAMP-PDE. Therefore, it appears that there is no correlation between the inhibition of type III cAMP-PDE and the secretion of TNFα and IL-1β in activated monocytes.

Rolipram and Ro20–1724, which are typically used as specific inhibitors of type IV cAMP-PDE, were very effective at reducing secreted TNFα and IL-1β. Rolipram was significantly more potent than Ro20–1724 for the inhibition of TNFα secretion, which correlates with their reported differences in IC_{50} values for type IV cAMP-PDE (see table 1). Interestingly, the two compounds were equipotent at inhibiting secretion of IL-1β. CP-77,059, a novel potent PDE IV inhibitor that is chemically distinct from rolipram and Ro20–1724 (Lowe *et al.*, 1991), was the most potent inhibitor in our cytokine assays.

To determine whether differences in cAMP levels correlated with inhibition of cytokine release, we chose IBMX, CI930 and rolipram as representative inhibitors from each of the above PDE categories for further analysis. These three inhibitors were compared directly over a wide range of doses for their ability to elevate cAMP and to inhibit TNFα and IL-1β. Rolipram elevated cAMP levels substantially more (2–3 times basal levels) than IBMX or CI930 (fig. 2A). Roli-

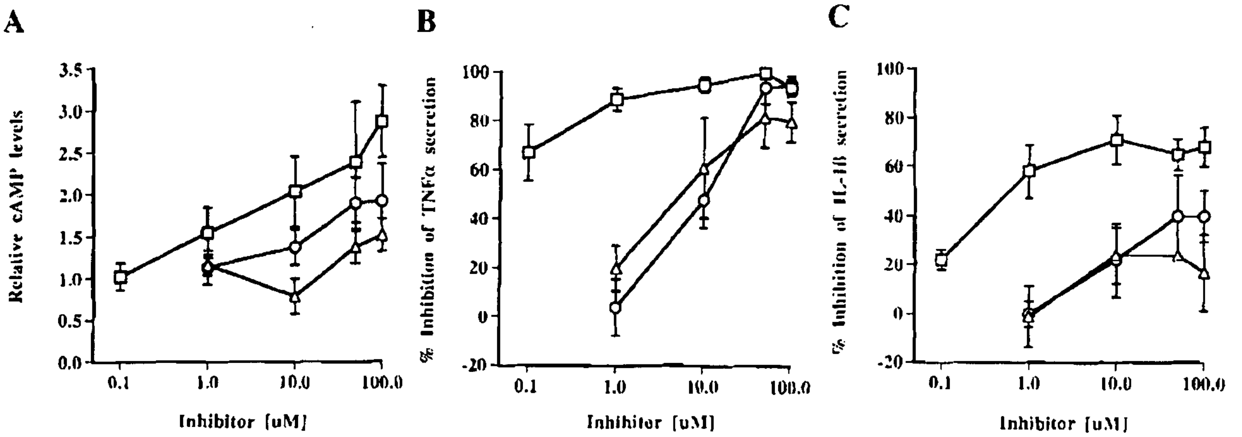

Fig. 2. Effect of rolipram, of CI930 and of IBMX on cAMP levels and cytokine secretion in LPS-stimulated human monocytes. A) Total cAMP levels (supernatants and cells lysed with 0.1% Triton X-100) were measured as described in "Materials and Methods." Duplicate 96-well plates from four donors were cultured with increasing concentrations of rolipram (□), CI930 (△) or IBMX (○) as described for cytokine measurements in B and C (see also "Materials and Methods"), but assays were terminated 15 min after the addition of LPS. cAMP levels are expressed as relative values, setting cAMP levels of LPS-stimulated controls as 1.0. LPS treatment did not significantly elevate cAMP levels compared to unstimulated cells (1.45 ± 0.70 *vs.* 1.35 ± 0.65 pmol cAMP/10^6 cells, respectively; $P < .05$). B and C) Percent inhibition of TNFα (B) or IL-1β (C) secretion is shown after the addition of increasing concentrations of rolipram (□), CI930 (△) or IBMX (○). LPS-stimulated control values from the same four donors used for cAMP measurements were 1.7 ± 0.5 ng/10^6 monocytes for TNFα and 10.0 − 3.5 ng/10^6 monocytes for IL-1β. Basal values were at or below the detection level of the ELISA kits (see fig. 1). The effects of rolipram on cAMP levels, TNFα and IL-1β were statistically significantly different ($P < .05$) from the effects of IBMX or CI930 on these three responses by analysis of variance (Snedecor, 1956).

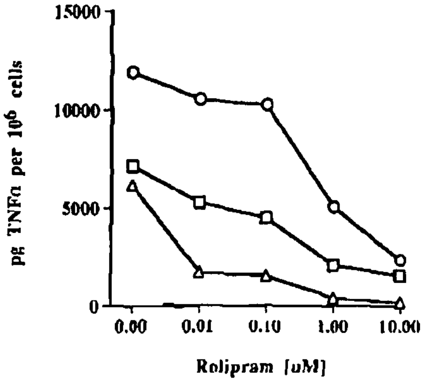

Fig. 3. Effect of 10 nM PGE$_1$ and of 1 μM indomethacin on rolipram inhibition of TNFα secretion by LPS-stimulated human monocytes. Adherent monocytes were treated with the indicated concentrations of rolipram in the absence (□) or presence of PGE$_1$ (△) or indomethacin (○) for 15 min before stimulation with 1 μg/ml of LPS. Supernatants were harvested at 18 hr to quantitate TNFα (pg/10^6 cells) by ELISA assay. The dose-response curve represents one donor, but similar results were observed in one (indomethacin) or five (PGE$_1$) additional donors.

pram was also the best compound to inhibit secretion of TNFα (fig. 2B) or IL-1β (fig. 2C) and was effective at concentrations as low as 0.1 μM. These results suggest that monocytes utilize type IV cAMP-PDE rather than type III cAMP-PDE to regulate cytokine secretion.

Effects of PGE$_1$ and of indomethacin on inhibition of TNFα by rolipram. If rolipram regulates cytokine secretion in monocytes through changes in cAMP levels, the addition of PGE$_1$ to raise cAMP levels should increase the effectiveness of rolipram. This was indeed the case. The IC$_{50}$ value for rolipram-mediated inhibition of TNFα decreased from 300 nM to 5 to 10 nM in the presence of 10 nM PGE$_1$ (fig. 3). Conversely, when indomethacin (1 μM) was added to prevent synthesis of prostaglandins and subsequent cAMP production, the IC$_{50}$ value increased, shifting the dose-response curve to the right (fig. 3). These findings support the hypothesis that the effects of rolipram are mediated through cAMP.

Effects of cAMP-elevating agents on cell-associated TNFα and IL-1β. All of the reagents capable of elevating cAMP levels in LPS-stimulated monocytes reduced the level of TNFα and IL-1β in the supernatants. To determine whether these agents were affecting only the secretion of these cytokines or their synthesis as well, we measured the amount of cell-associated TNFα and IL-1β in monocytes treated with LPS in the presence of cAMP-elevating agents. Only a minor proportion of TNFα was detectable as a cell-associated form (data not shown), but more than 50% of IL-1β remained associated with LPS-stimulated monocytes (compare fig. 4A and 4B), although this ratio varied considerably among different donors. Under our experimental conditions, neither rolipram, dbcAMP nor PGE$_1$ diminished the amount of detectable IL-1β that was cell-associated (fig. 4B), although these agents clearly reduced IL-1β in the cell supernatants (fig. 4A). As a positive control, 1 μM dexamethasone was used to inhibit cell-associated IL-1β in these experiments (92 ± 4% inhibition relative to controls with n = 3, data not shown).

Effects of dbcAMP, of PGE$_1$ and of rolipram on mRNA levels for TNFα and IL-1β in LPS-stimulated monocytes. To determine whether rolipram regulates cytokine expression at a transcriptional level, monocytes were treated with LPS in the presence or absence of 1 μM PGE$_1$, 100 μM dbcAMP or varying concentrations of rolipram. mRNA was purified from these cells and analyzed by Northern blot (fig. 5). Although there was a significant amount of IL-1β mRNA but not of TNFα mRNA in the control samples, LPS induced both TNFα and IL-1β mRNA approximately 10-fold. Because these cells were isolated by adhesion and therefore might be partially activated, the appearance of mRNA for IL-1β was not unexpected (Dinarello *et al.*, 1987). In accordance with previous results (Renz *et al.*, 1988; Serkkola *et al.*, 1992), dbcAMP or PGE$_1$ significantly reduced accumulation of TNFα mRNA (70%–90%), whereas accumulation of IL-1β mRNA was moderately elevated (25%–45%). Rolipram mimicked the effects of these cAMP-elevating agents in a dose-dependent manner, which suggested that the effects of this cAMP-PDE inhibitor were mediated through cAMP.

cAMP-PDE activity in human monocytes. The above results showed that type IV-specific cAMP-PDE inhibitors effectively inhibited the appearance of cytokines in the supernatant from LPS-stimulated monocytes. This suggested that type IV-specific cAMP-PDE is the predominant PDE type in LPS-stimulated monocytes. In fact, a rolipram-sensitive (K$_i$ = 1.5 μM), high-affinity cAMP-PDE had previously been isolated from cytosolic fractions of frozen human blood

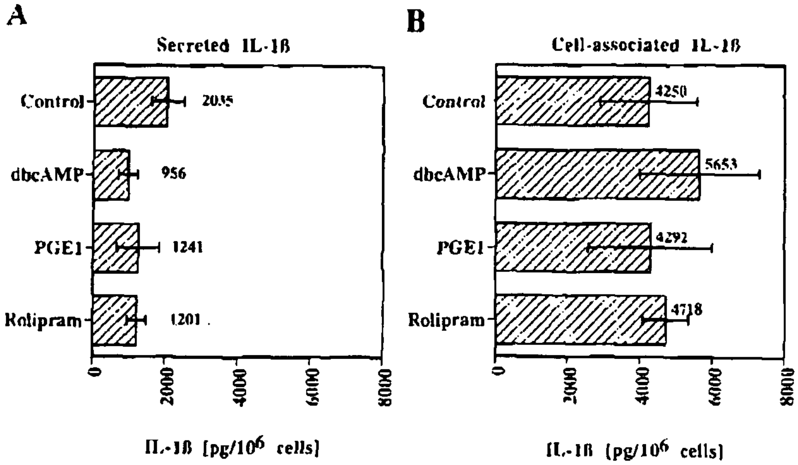

Fig. 4. Effect of 100 μM dbcAMP, of 1 μM PGE$_1$ and of 50 μM rolipram on cell-associated or secreted IL-1β in LPS-stimulated human monocytes. Adherent monocytes were treated with or without 100 μM dbcAMP, 1 μM PGE$_1$ or 50 μM rolipram for 16 hr in the presence of 1 μg/ml of LPS. IL-1β in the supernatants (A) and in the lysates (B) was assayed by ELISA as described in "Materials and Methods." Data are expressed as pg of IL-1β/10^6 cells and are averages from four different donors. Effects of dbcAMP and rolipram on secreted IL-1β were statistically significant ($P < .05$) by analysis of variance (Snedecor, 1956).

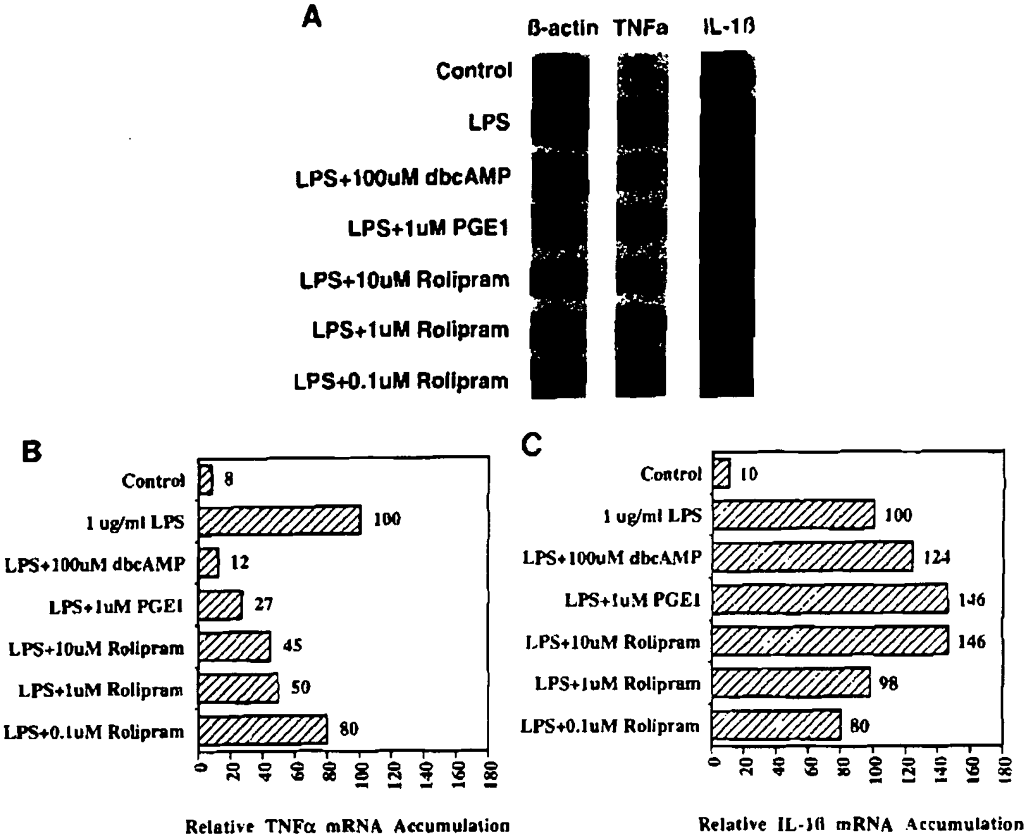

Fig. 5. TNFα and IL-1β steady-state mRNA levels in LPS-stimulated monocytes treated with dbcAMP, PGE_1 and rolipram. A) PolyA+ mRNA was analyzed by Northern blot analysis from LPS-stimulated monocytes treated with no inducers (control), 1 μg/ml LPS alone or LPS with 100 μM dbcAMP, 1 μM PGE_1 or rolipram at 10, 1 or 0.1 μM. The left, middle and right panels show the autoradiogram of the blot after probing with human β-actin, TNFα and IL-1β specific probes, respectively (see "Materials and Methods"). B and C) The level of radioactivity for each TNFα and IL-1β band was quantitated and normalized to the corresponding β-actin band (see "Materials and Methods"). The values for TNFα (B) and IL-1β (C) were plotted for each treatment as a relative percent of mRNA accumulated compared to the 1 μg/ml LPS-treated sample (100%). Data shown are from a single donor, but monocytes from two additional donors yielded similar results.

monocytes (Torphy *et al.*, 1992). To isolate cAMP-PDE activity and determine whether it is predominantly of the type IV group, fresh monocytes were obtained by elutriation and permeabilized with digitonin to release cytosolic proteins. This procedure extracted more than 90% of cAMP-PDE activity from these cells. Table 2 shows that 70% of cAMP-PDE activity in the digitonin extract was inhibited by 10 μM rolipram. When cilostamide (K_i = 0.02 μM for type III PDE) or cGMP was used at concentrations expected to inhibit type III PDE activity completely, enzyme activity in our preparations was reduced by only 20%. These data confirm that most of the cytosolic PDE in monocytes is type IV cAMP-PDE (Thompson *et al.*, 1976; Torphy *et al.*, 1992).

TABLE 2

Effect of selective modulators on cAMP-PDE activity in monocyte cytosol[a]

Treatment	cAMP Hydrolyzed[b]	% Inhibition
Buffer	74 ± 12	0
Rolipram	23 ± 5	72 ± 5
Cilostamide	65 ± 3	26 ± 6
cGMP	71 ± 3	19 ± 5

[a] cAMP-PDE activity in digitonin extracts from elutriated monocytes was assayed in the absence (buffer) or presence of 10 μM rolipram, 10 μM cilostamide or 10 μM cGMP (see "Materials and Methods"). The values shown for cAMP-PDE activity are averages ± S.E.M. from three different donors, representing three independent experiments. Inhibition of cAMP-PDE activity by rolipram was statistically significantly different ($P < .05$) from inhibition by either cilostamide or cGMP by analysis of variance (Snedecor, 1956).

[b] fmol of cAMP hydrolyzed/min/mg protein.

Discussion

In order to determine whether human blood monocytes possessed a cAMP-PDE activity that could modulate cAMP levels and regulate cytokines, we investigated the potential for specific PDE inhibitors to lower TNFα and IL-1β production in LPS-stimulated human peripheral blood monocytes. Raising cAMP levels directly by adding dbcAMP to LPS-stimulated monocytes reduced the appearance of both TNFα and IL-1β in the supernatants. At all concentrations of dbcAMP tested, TNFα was reduced more effectively than IL-1β. Activation of adenylate cyclase by PGE_1 was effective in raising the levels of intracellular cAMP in LPS-stimulated monocytes and was as effective as dbcAMP in inhibiting the appearance of secreted TNFα and IL-1β. The inhibition by PGE_1 was dose-dependent and was more effective for TNFα than for IL-1β.

PDE inhibitors were also capable of raising intracellular cAMP levels and of inhibiting TNFα and IL-1β in the supernatants of LPS-stimulated monocytes. The two chemically related compounds rolipram and Ro20–1724, which are typically used to target type IV cAMP-PDE selectively, were potent inhibitors of cytokine release. CP-77,059, another inhibitor of type IV cAMP-PDE, which is chemically distinct from rolipram, was the most potent compound among all the inhibitors tested in our assays. The order of potency for inhibition of TNFα secretion for these type IV cAMP-PDE inhibitors correlated with their reported IC$_{50}$ values for PDE IV inhibition (see table 1). In contrast, compounds specific for type III cAMP-PDE (C1930 and cilostamide) were ineffective in reducing the secretion of IL-1β and inhibited the release of TNFα only at high concentrations where these compounds lose their selectivity. The nonselective PDE inhibitors theophylline and pentoxifylline significantly diminished TNFα in the supernatants but were effective only at >50 μM. IBMX was consistently the most potent of the nonselective inhibitors in reducing supernatant levels of TNFα and of IL-1β, but IBMX was still 100 times less effective than rolipram in these assays. The above inhibitor profiles agree well with similar recent reports (Molnar-Kimber *et al.*, 1992; Semmler *et al.*, 1993) and indicate that monocytes are similar to neutrophils and basophils in that their activation can be regulated by type IV cAMP-PDE (Nielson *et al.*, 1990; Peachell *et al.*, 1992; Verghese *et al.*, 1989). This enzyme also appears to be the predominant enzyme present in monocytes, because rolipram was much more effective than cilostamide in inhibiting cAMP degradation in cytosol prepared from fresh or frozen peripheral blood monocytes (our data and Torphy *et al.*, 1992).

Significant inhibition of TNFα or IL-1β release occurred at drug concentrations where increases in total cAMP levels were barely detectable. For example, TNFα secretion was inhibited 60% by 0.1 μM rolipram and 50% by 0.01 μM PGE$_1$ without a significant change in cAMP level. Similar observations have been reported in other systems, such as Leydig cells and granulosa cells, where substantial increases in steroid biosynthesis occur at concentrations of hormones that do not elicit detectable changes in levels of cAMP (Pereira *et al.*, 1987). It is possible that localized elevations of cAMP that are not detectable with current methodologies are sufficient to downregulate TNFα and IL-1β, but we cannot exclude cAMP-independent mechanisms. Nevertheless, the finding that three different methods of raising intracellular cAMP levels had similar effects on these two cytokines strongly implicates cAMP as the mediator of these effects in human monocytes.

cAMP-elevating agents appear to regulate IL-1β production in LPS-stimulated monocytes differently than they do TNFα production. It is shown here that elevating cAMP levels in many different ways does not affect the overall synthesis of IL-1β but only diminishes the secreted amount. This agrees with recently published data on the effects of cAMP levels on IL-1β production by human monocytes (Viherluoto *et al.*, 1991), but it contrasts with earlier reports that concluded that PGE$_2$ serves as a negative-feedback regulator for IL-1β production in these cells as well as in murine macrophages (Dinarello *et al.*, 1987; Brandwein, 1986). However, many of the earlier studies used cell proliferation assays rather than immunological methods to quantitate IL-1β and are therefore not directly comparable.

Previous studies have showed that cAMP reduces TNFα steady-state mRNA levels without affecting IL-1β mRNA accumulation (Knudsen *et al.*, 1986; Scales *et al.*, 1989). To determine whether this correlation is observed in the presence of type IV cAMP-PDE inhibitors, we performed Northern blots to quantitate TNFα and IL-1β mRNA levels in monocytes treated with LPS and rolipram. Rolipram dramatically reduced mRNA accumulation for TNFα but increased steady-state IL-1β mRNA levels. Similar enhancement of mRNA levels for IL-1β has been observed in monocytes treated with dbcAMP or PGE$_2$ (Serkkola *et al.*, 1992; Sung and Walters, 1991). This again suggests that the effects of rolipram are mediated through changes in cAMP levels. Like other cAMP-elevating agents, rolipram apparently regulates the synthesis of TNFα at a transcriptional level, whereas the expression of IL-1β clearly is regulated both transcriptionally and post-translationally. Although it is not shown here how cAMP levels affect IL-1β production post-transcriptionally, our data support those of others that indicate that cAMP interferes with secretion of IL-1β rather than with other steps in the biosynthetic pathway (Scales *et al.*, 1989; Viherluoto *et al.*, 1991).

Regardless of whether the regulation is transcriptional or post-transcriptional, inhibitors of type IV cAMP-PDE are as effective as other cAMP-elevating agents in diminishing levels of TNFα and IL-1β in supernatants from LPS-activated monocytes. Selective inhibitors of type IV cAMP-PDE could thus provide a novel way to combat acute and chronic inflammatory diseases wherein these cytokines play important roles. The finding that multiple genes code for rolipram-sensitive type IV PDE isozymes raises the possibility of discovering more highly selective inhibitors that may differentiate among isozymes within this class (Beavo and Reifsnyder, 1990; Conti and Swinnen, 1990). The existence of these genes, which are differentially expressed in somatic and germ line cells of rat testis (Conti and Swinnen, 1990), suggests that tissue-specific inhibitors of type IV cAMP-PDEs could be developed as specific therapeutic agents.

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